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Nanoscale segregation of actin nucleation and elongation factors determines dendritic spine protrusion

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: David del Alamo

1st Editorial Decision 16 May 2014

Thank you for the submission of your manuscript entitled "Nanoscale segregation of branched F-actin nucleation and elongation determines dendritic spine shape". We have now received the reports from the referees that were asked to evaluate your study, which I copy below.

As you can see from their comments, all three referees are absolutely supportive of the publication of your manuscript in The EMBO Journal. For the most part, they express certain concerns with the last part of the manuscript, which in their opinion becomes rather speculative. In this sense, referee #2 suggests some experiments to further substantiate your conclusions, and both referee #1 and #3 believe that certain ideas should be toned down in the discussion based on the evidence presented. It is interesting -and a point that needs to be addressed- that all three referees agree on the difficulty to extract a physiologically meaningful conclusion of the constitutively active Rac1 expression experiment, and both referees #2 and #3 suggest alternative, easier to interpret assays involving chemical LTP.

Given these positive evaluations, I would like to invite you to submit a revised version of the manuscript. It is 'The EMBO Journal' policy to allow a single round of major revision only, which should be submitted within the next three months. In this regard, do not hesitate to contact me if you have any question, need any further input or anticipate any problems along the revision process. Should you foresee a problem in meeting the three-month deadline, please let us know in advance

and we may be able to grant an extension.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, we would appreciate if you contact me as soon as possible upon publication of any related work in order to discuss how to proceed.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1:

The manuscript by Chazeau et al. 'Nanoscale segregation of branched F-actin nucleation and elongation determines dendritic spine shape' deals with the fundamental question of how actin is nucleated in dendritic spines.
Although it is well recognized that actin polymerization controls spine morphology and function, the exact mechanism how actin nucleation occurs in spines is still debated and not well understood. One hypothesis is that the mechanism is similar to the one observed in classical cell protrusions such as the lamellipodia.
This is the starting point of the work by Chazeau et al,, trying to elucidate whether this hypothesis is true. The authors use cultured neurons transfected with the respective fluorescent proteins and super resolution microscopy in order to determine the relation of NPFs (nucleation) to Arp2/3 (branching) on a rather challenging spatial resolution. Given the small size of a spine this work is a valuable effort to better understand actin polymerization in spines.
Although for someone outside of the field the excitement about the data might not be immediately visible, the presented work is of great interest to neurobiologist and cell biologists. The manuscript provides solid evidence that actin polymerization in dendritic spines occurs by an unexpectedly different mechanism, when compared to lamellipodia. The PSD in spines appears to serve as an organizer or structural platform for NPFs such as the WAVE-complex - meaning that actin nucleation is initiated from here. Branching nucleation then appears to occur close from the PSD with elongation of filaments propagating outwards.
In my opinion the manuscript contains very important finding that will stimulate further experiments in the field. Obviously there are numerous interesting additional questions which arise from the work. Is the entire WAVE-complex localized to the PSD? And how is the dynamics of complex components in and out the PSD with respect to initiation of actin nucleation?

Nevertheless, there are a number of issues that need to be addressed by the authors. Main point: The first 2/3 of the manuscripts are beautifully done, towards the end of the manuscript the conclusions are becoming very 'bold' based on rather weak evidence. This part is moving on very thin ice.

Critique:

- the title is somewhat unfortunate and should be adjusted, branched F-actin nucleation is not part of the work and not really shown anywhere, only circumstantial conclusion because of the localization of Arp-components, also there is little on dendritic shape or ddynamics said in the manuscript ...
- similarly there are a number of incorrect or exaggerated statements and use of expressions throughout the manuscript such as 'F-actin branch points', 'F-actin branching', 'WAVE' etc.. This is not formally shown and Abi1 localization must not be set equal to WAVE or WAVE complex presence. There is plenty of evidence that in particular Abi is part of other complexes as well. The manuscript needs to be carefully revised concerning these point. This needs to be precise. It is otherwise misleading.
- the data in Fig. 3 and the conclusions are somewhat confusing to me, in principle ArpC5a looks

comparable to VASP and there is limited overlap with PSD95. This the authors take as support that nucleation is close to the PSD. And the rest of ArpC5a which is somewhere else is doing what? Why could this not lead to branched nucleation ?
- the part on Rac1 in spines seems rather shaky and fuzzy. A lot of spinning around the fact that Rac1 is obviously freely diffusing and can therefore bind to the WAVE-complex. So what? Am I missing here anything that is beyond obvious and expected observation ?
- the last chapter on dendritic spine enlargement is the weakest part. The authors are trying to explain finding upon overexpression of constitutively active Rac-mutants, which leads to enlarged spine structures. In the end the authors take this as an indication that enhanced synaptic activity might trigger WAVE delocalization and Arp2/3 activation. This is misleading. The neurons are transfected and then cultured for many days and at the end when the experiments are performed we are looking at neurons which have adapted for 10 days or so to constitutively active Rac1. This has nothing to do with 'stimulus triggered spine enlargement'. If they want to add anything here the authors must look at acute treatment of neurons (e.g.BDNF?!) and then look at the actin machinery.

Referee #2:

The paper by Chazeau et al. used a state-of-art superresolution technique to elucidate the dynamics of multiple actin interacting proteins within dendritic spine. It has very unique information and experiments are generally well conducted. This reviewer trusts that it is highly appropriate for publication in EMBO J.

There are a few points need to be clarified.

Tatavarty et al. and Frost et al. used a very similar technique of single particle tracking and found a rearward movement of actin. Also, Honkura used PA-GFP actin and observed a similar, though it was bulk imaging. Where is this discrepancy coming from?

Finger-like extensions the authors observe resembles what other people call "spinules". The identity of these two structures (or otherwise) should be carefully tested. Also, EM reconstruction studies of spines in vivo such as those by Harris seldom see such structure. So the frequent occurrence of the finger-like extension may be some artifact unique to the author's system.

Other comments.

1. Spine/protrusion of mEos2-VASP expressing neuron looks abnormal (Fig.2B). Molecular behavior in abnormal neuron would not be normal. Similarly, mEos2-Rac1-T17N expressing neuron also showed abnormal morphology (Fig. 6E).
2. Fig. 3A and C. In the provided images, it is hard to tell whether the dot like structure exist at the tip of the singer-like extension or within spine. As a matter of fact, dot-like signals are all over. Also, what it the structure extending upward from in Fig 3A green channel?
3. Fig. 4H. It is a little surprising that cytosolic-mEOS2 shows a significant immobile fraction. Could it be an error of tracking? Protein aggregation? Has the authors tried mEOS3?
4. It would be better to show the data using Rac inhibitors to conclude Rac motility, because Rac-N17 expressing neuron has no mushroom spine (Fig.6E). Expecting result would be that inhibition Rac-GTP by EHT1864 increases free diffusion fraction of Rac1.
5. In spine enlargement experiment, they used over expression of active Rac or Shank3 (Fig.7). But as it is well established that chemical-LTP protocol induces spine enlargement in dissociated neuron (Fortin et al., 2010; Bosch et al., 2014), chem.LTP would be more physiologically relevant system than over-expression of signaling molecules. Authors can compare molecular motility in a same spine at basal or potentiated condition.

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings

Chazeau et al. study advance our understanding on the actin dynamics in dendritic spines, how actin polymerization can be translated into proper spine head morphology. The main finding is that actin is nucleated next to PSD but polymerization of the filaments occurs at the tip of finger-like protrusions. Especially the idea that nucleation locates stably next to PSD is new. Model is well supported by the presented data. The idea that neuron activation induced changes are achieved through the WAVE mobilization from the PSD area is good but I don't think the experiments used here are really relevant to support this idea.
Technically manuscript seems to be solid, special thanks for presenting the normal resolution images next to super resolution images. Those images make it easier to see how dendrites look like for readers who are not used to super resolution images. They also nicely highlight how super resolution images differ from normal resolution images.

Specific major concerns essential to be addressed to support the conclusions

1) Spine enlargement model is interesting but the problem is that enlargement is induced here by over-expression of constitutively active Rac1 or over-expression of Shank3. It is possible that when these proteins are over-expressed they start to activate WAVE in "wrong" locations resulting for example lamellipodia on the dendrites, which is obviously not the normal situation. It is also unclear if these over-expressions have anything to do with neuron activation induced spine enlargement. Based on the recent article from Hayashi laboratory (Bosch et al., Neuron 2014), expression levels of PSD proteins, such as Homer1b and Shank1b, increases 60 minutes after the fast spine enlargement and it is plausible that in contrast to induce actin rearrangements, they stabilize the actin network. Therefore it is questionable what the over-expression experiments can really tell us. If authors want to test neuron activation induced spine head growth they should perform some neuron activation, easiest might be the chemical LTP. Although chemical LTP is not the most fanciest method to activate neurons it would be anyway more relevant way to address this question than activating Rac1 or over-expressing Shank3.

Minor concerns that should be addressed

1) "In the brain, most excitatory post-synapses are small membrane extensions called dendritic spines."?
I would not define dendritic spines as post-synapses. Dendritic spines are just protrusions where synapses locate.

2) Could authors explain the difference with the localization presented for WAVE complex in this manuscript and that presented for WAVE-1 in Soderling et al, J. Neuroscience 2007 (Fig. 2E)?

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

1) "Control of F-actin length and turnover in spines is then ensured by elongating factors such as formins (Hotulainen et al, 2009) and VASP (Lin et al, 2010), capping proteins (Fan et al, 2011; Korobova & Svitkina, 2010), and F-actin severing proteins such as ADF/cofilin (Gu et al, 2010; Rust et al, 2010; Bosch et al, 2014)."
I understand how I should read this sentence but there is a possibility to misread it so that capping proteins and and ADF/cofilins are also elongating factors. Maybe good to rephrase that.

2) "Consequently, Arp2/3 and actin monomers are incorporated in branched F-actin networks growing inward from the lamellipodium tip."
I would write that F-actin network GROW toward/against the tip of lamellipodium and FLOW inward.

3) It is not clear for me which IRSp53 domains the small circles represent. If they represent IRSp53

membrane binding domains (F-BAR), why are they directed outwards from membrane and not against the membrane?

1st Revision - authors' response 13 August 2014

Referee #1:

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Although for someone outside of the field the excitement about the data might not be immediately visible, the presented work is of great interest to neurobiologist and cell biologists. The manuscript provides solid evidence that actin polymerization in dendritic spines occurs by an unexpectedly different mechanism, when compared to lamellipodia. The PSD in spines appears to serve as an organizer or structural platform for NPFs such as the WAVE-complex - meaning that actin nucleation is initiated from here. Branching nucleation then appears to occur close from the PSD with elongation of filaments propagating outwards.

In my opinion the manuscript contains very important finding that will stimulate further experiments in the field. Obviously there are numerous interesting additional questions which arise from the work. Is the entire WAVE-complex localized to the PSD? And how is the dynamics of complex components in and out the PSD with respect to initiation of actin nucleation?

Nevertheless, there are a number of issues that need to be addressed by the authors. Main point: The first 2/3 of the manuscripts are beautifully done, towards the end of the manuscript the conclusions are becoming very 'bold' based on rather weak evidence. This part is moving on very thin ice.

Critique:

- the title is somewhat unfortunate and should be adjusted, branched F-actin nucleation is not part of the work and not really shown anywhere, only circumstantial conclusion because of the localization of Arp-components, also there is little on dendritic shape or dynamics said in the manuscript.

We thank the reviewer for those constructive questions and comments.

We agree with the reviewer that we are not directly studying branched F-actin nucleation events. Nevertheless, most of our experiments focus on branched F-actin regulators (Rac1, WAVE complex subunits, IRSp53). Even FMNL2 has been demonstrated to cooperate with Arp2/3 to regulate the formation of branched F-actin networks (Block *et al*., Curr. Biol. 2012). This is why we used ‘branched F-actin nucleation’ in the original title. However, in complete agreement with our data we are now referring to the proteins (nucleation promoting factors (NPFs) and elongation factors) and not to the functions (nucleation and elongation).

Although the manuscript is not completely focused on the shape of dendritic spines, our results support the hypothesis that the specific segregation of NPFs and elongators in spines is responsible or associated with the formation of finger like protrusion in spines. In addition, our results provide evidence that long lasting modification of Rac1 or Shank3 functions, which trigger spine enlargement, are correlated with delocalization of the WAVE complex from the PSD. Thus, we think that our manuscript does include findings about the shape of spines.

Nevertheless, we are not against changing the title of the manuscript to take into account these comments of the reviewer. We propose to replace 'Nanoscale segregation of branched F-actin nucleation and elongation determines dendritic spine shape' by **'Nanoscale segregation of actin nucleation and elongation factors determines dendritic spine protrusion'.**

In the Result section, page 7, we also replaced the title 'F-actin elongations delocalize outwards from the PSD while Arp2/3 nucleations occurs close to the PSD' by 'F-actin elongators delocalize outwards from the PSD while nucleation factors localize at the PSD'.

- similarly there are a number of incorrect or exaggerated statements and use of expressions throughout the manuscript such as 'F-actin branch points', 'F-actin branching', 'WAVE' etc.. This is not formally shown and Abi1 localization must not be set equal to WAVE or WAVE complex presence. There is plenty of evidence that in particular Abi is part of other complexes as well. The manuscript needs to be carefully revised concerning these point. This needs to be precise. It is otherwise misleading.

We thank the reviewer for pointing out those exaggerated statements. We agree with the reviewer that Arp2/3 complex localization is not always equivalent to the localization of Arp2/3 branches. This point will be discussed further in response to the following comment. In the revised version of the manuscript we replaced 'F-actin branch points' and 'F-actin branching' as follows:

- In the abstract we replaced 'Arp2/3 branch points are immobile and surround the PSD' by 'Arp2/3 complexes associated with F-actin are immobile and surround the PSD'.
- Page 5 we replaced 'F-actin branch points are not exhibiting a concerted rearward flow in spines' by 'F-actin and the Arp2/3 complex are not exhibiting concerted rearward flow in spines'.
- Page 6 we replaced 'Thus, the slow rate of F-actin branch points implies that their elongation will lead to efficient pushing forces triggering membrane protrusions and spine motility' by 'Thus, the slow rate of F-actin and Arp2/3 complex movements implies that F-actin elongation will lead to efficient pushing forces triggering membrane protrusions and spine motility'.
- Page 8 we replaced 'The co-localization of PSD-95, WAVE and IRSp53 at the center of the spine, together with the slow rate and weak polarity of F-actin branch point movements (Fig 1) suggested that Arp2/3 nucleation is not occurring at protrusion tips in spines.' by 'The co-localization of PSD-95, the WAVE complex and IRSp53 at the center of the spine, together with the slow and unpolarized motions of branched F-actin networks (Fig 1) suggested that Arp2/3 nucleation is not occurring at protrusion tips in spines.'.
- Page 14 we replaced 'Elongation from immobile branched points close to the PSD will not generate a concerted, fast rearward flow of Arp2/3 and F-actin, consistent with our results and previous studies on actin dynamics' by 'Elongation from immobile Arp2/3 close to the PSD will not generate a concerted, fast rearward flow of Arp2/3 complex and F-actin, consistent with our results and previous studies on actin dynamics'.
- Page 31 we replaced 'Arp2/3 complex and associated F-actin branches are not moving rearward in spines' by 'Arp2/3 complex and F-actin movements are not polarized in spines'.
- Page 36 in the legend of Figure 8 we replaced 'However, growing F-actin barbed ends (+) are located at the tip of membrane protrusions, where elongation of F-actin branches is catalyzed by VASP and FMNL2.' by 'However, growing F-actin barbed ends (+) are located at the tips of membrane protrusions, where elongation of F-actin is catalyzed by VASP and FMNL2.'.
- Page 36 in the legend of Figure 8 we replaced 'In this model, elongation from immobile branched points close to the PSD will not generate a concerted fast rearward flow of Arp2/3 and F-actin in spines.' by 'In this model, elongation from immobile Arp2/3 close to the PSD will not generate a concerted fast rearward flow of Arp2/3 complex and F-actin in spines.'.
- Page 41 in the legend of Supplementary Figure S9 we replaced 'Transient zones of immobilization (arrow) continually relocate and correspond to F-actin branches.' by 'Transient zones of Arp2/3 immobilization (arrowhead) continually relocate within spines.'.

We agree with the reviewer that Abi1 is not completely equivalent to the WAVE complex. Several studies showed that Abi can act separately from the WAVE complex (Disanza *et al*., Nat Cell Biol 2004; Proepper *et al*., EMBO J 2007; Park *et al*., J Neurosci 2012). This is also true for other

subunits of the WAVE complex including Sra (Anitei *et al*., Nat Cell Biol 2010; De Rubeis *et al*., Neuron 2013). Therefore, in the Result section, we referred to Abi1 when describing experiments using Abi1. Nevertheless, many studies demonstrated Abi to be part of the stable pentameric WAVE complex (Gautreau *et al*., PNAS 2004; Innocenti *et al*., NCB 2004) along with Sra, Nap, Brk, and Wave.

Therefore, we think that we can hypothesize that the localization of Abi at least in part reflects the localization of the WAVE complex. Supporting this hypothesis, endogenous Wave (1 and 2) proteins, imaged using dSTORM, also displayed clusterization at the center of the spine (Supplementary Fig S4A).

For the revised manuscript, and to further support the nanoscale localization and dynamics of the WAVE complex in spines, we performed additional experiments on Nap1, another subunit of the WAVE complex.

Live super-resolution intensity images showed that mEOS2-Nap1 was not localized at protruding tips, but clustered into a single central domain within dendritic spines (see Supplementary Fig S3B). Like mEOS2-Abi1, dual-color PALM/dSTORM experiments demonstrated that mEOS2-Nap1 detections formed a domain having the same size and overlapping with the PSD-95 domain (see Fig 3D, E and Supplementary Fig S5B). In addition, we performed high-frequency sptPALM acquisition (20 Hz) to characterize the diffusive properties of mEOS2-Nap1. Like mEOS2-Abi1, distributions of D for mEOS2-Nap1 were shifted towards faster diffusions compared to F-actin and Arp2/3 (see Supplementary Fig S5C-G). Both in shafts and spines, the fraction of immobilized mEOS2-Abi1 and mEOS2-Nap1 were lower, and the fractions of confined and free-diffusion were larger (see Supplementary Fig S5F). Furthermore like mEOS2-Abi1, many confined and immobile mEOS2-Nap1 trajectories were detected in the same area (see Supplementary Fig S5D).

Thus, these results further support that the WAVE complex is retained in a confinement zone forming a long-lasting domain overlapping with the PSD.

To take into account the comment of the reviewer and our additional data we modified and added in the manuscript the following sentences:

- Page 8 we added:
  'Since Abi1 could also be part of other protein complexes (Proepper *et al*., 2007), we performed experiments using Nap1, another subunit of the WAVE complex, to further support the nanoscale organization of the WAVE complex. Live super-resolution intensity images and dual-color PALM/dSTORM experiments demonstrated that mEOS2-Nap1, like mEOS2-Abi1, was not localized at protruding tips, but also clustered into a single central domain having the same size and overlapping with PSD-95 (Figure 3D and E, Supplementary Fig S3 and S5).'
- Page 10 we replaced in the title 'The WAVE complex and IRSp53 are associated with confined components of the PSD' by 'Abi1, Nap1 and IRSp53 are associated with confined components of the PSD'.
- Page 10 we replace 'To study the diffusion properties of the wave complex in spines, we performed sptPALM experiments using mEOS2-Abi1 (Fig 5A and B).' by 'To study the diffusion properties of WAVE complex subunits in spines, we performed sptPALM experiments using mEOS2-Abi1 and mEOS2-Nap1 (Fig 5A and B; Supplementary Fig S5C and D; Supplementary Table S1).'.
- Page 10 replaced 'Thus, the WAVE complex is retained in a confinement zone forming a long-lasting domain overlapping with the PSD.' by 'Thus, those results suggest that the WAVE complex is retained in a confinement zone forming a long-lasting domain overlapping with the PSD.'.
- Page 10 and 11 we added to Abi1, Nap1: replacing all 'Abi1' by 'Abi1 and Nap1'.
- Page 12 we replaced the title 'Dendritic spine enlargement is associated with delocalization of WAVE from the PSD' by 'Dendritic spine enlargement is associated with delocalization of Abi1 from the PSD'.
- Page 14 we replaced 'Second, the WAVE complex and IRSp53 are retained close to the PSD.' by 'However, the WAVE complex subunits Abi1 and Nap1 along with IRSp53 are retained close to the PSD.'.
- Page 14 we replaced 'Consistent with retention close to the PSD, WAVE, IRSp53 and Arp2/3 are directly and indirectly binding to PSD components, including CAMKII, Shank1, Shank3 and PSD-95 (Hering & Sheng, 2003; Bockmann *et al*, 2002; Proepper *et*

al, 2007; Han *et al*, 2013; Choi *et al*, 2005; Park *et al*, 2012).' by 'Consistent with retention close to the PSD, Abi1, IRSp53 and Arp2/3 are directly and indirectly binding to PSD components, including CAMKII, Shank1, Shank3 and PSD-95 (Hering & Sheng, 2003; Bockmann *et al*, 2002; Proepper *et al*, 2007; Han *et al*, 2013; Choi *et al*, 2005; Park *et al*, 2012).'.

- Page 33 we replaced in the figure title 'WAVE, IRSp53 and PSD-95 are confined at the PSD' by 'Abi1, Nap1, IRSp53 and PSD-95 are confined at the PSD'
- Page 35 we replaced in the figure title 'Rac1 enhanced activity and Shank3 over-expression delocalizes WAVE from the PSD' by 'Rac1 enhanced activity and Shank3 over-expression delocalizes Abi1 from the PSD'
- Page 42 we replaced in the figure title 'VASP and WAVE localize at the tips of lamellipodia-like structures induced by constitutively active Rac1.' by 'VASP and Abi1 localize at the tips of lamellipodia-like structures induced by constitutively active Rac1.'

To avoid any misleading conclusion about the WAVE complex and its subunits we made the following changes in the final manuscript:

- In the entire manuscript we replaced all 'WAVE' by 'WAVE complex', or by 'WAVE complex subunits' or 'WAVE complex subunits Abi1 and Nap1'.

- the data in Fig. 3 and the conclusions are somewhat confusing to me, in principle ArpC5a looks comparable to VASP and there is limited overlap with PSD95. This the authors take as support that nucleation is close to the PSD. And the rest of ArpC5a which is somewhere else is doing what? Why could this not lead to branched nucleation ?

Our data demonstrated that subunits of the WAVE complex (Abi1 and Nap1) and IRSp53 form a central domain co-localized with the PSD. While VASP and FMNL2 move outwards from the PSD with protrusion tips. These conclusions are supported by Figure 2, 3 and Supplementary Figure S3. Compare Figure 2B, C (VASP/FMNL2) with Supplementary Figure S3 (Abi1 and Nap1) and Supplementary Figure S6 (IRSp53).

We agree with the reviewer that the localization of Arp2/3 is less well defined. It is not forming a central domain co-localized with the PSD. Nevertheless, Arp2/3 nano-domains are partially over-lapping with PSD-95 (see Figure 3C). In addition, ArpC5A is not concentrated like VASP or FMNL2 at the tip of membrane protrusions, compare Figure 2B, C (VASP/FMNL2) with Sup Figure S3C (the outline of the spine clearly show finger-like extension devoid of Arp2/3 nano-domains). Those differences are supported by the statistical analysis of our experiments (Fig 3E and Supplementary Fig S3D).

We want to point out that differences in localization between ArpC5A and VASP are more pronounced in live experiments (Supplementary Fig S3) compared to fixed neurons (Fig 3). This could be explained by the fact that in live experiments, fast free-diffusing protein fused to mEOS2 (membrane or cytosolic) will outline the spine shape while confined and immobile mEOS2 will accumulate in sub-spine domains; the central domain for WAVE complex subunits, the protrusion tips for VASP/FMNL2, and Arp2/3 nano-domains. However, in fixed experiments (dSTORM or dSTROM/PALM), the fast free-diffusing proteins will be immobilized, due to fixation, in locations that do not correspond to their real accumulation in specific sub-spine domain. Thus in fixed experiments those 'false immobile' proteins add noise to the 'real confined/immobilized' proteins selectively observed in live experiments. We think that those 'non-specific' immobilizations could contribute to the less pronounced differences between Arp2/3 and VASP in fixed experiments.

To corroborate that Arp2/3 localizations are not concentrated at protrusion tips like VASP, we performed additional dual-color PALM/dSTORM experiments (see attached Referee Figure 2; PALM for mEOS2-ArpC5A and dSTORM for VASP-GFP labelled with an anti-GFP Alexa antibody). Those experiments showed that, within the same spine, the localization of the Arp2/3 complex is distinct from the localization of VASP. ArpC5A is often more central to the spine and surrounded by VASP. Therefore, the localization of Arp2/3 is comparable but distinct from the localization of VASP/FMNL2.

There was also a problem of representation in PALM/dSTORM experiments (Figure 3, 7, S5, S6 and S10). Using 8 bit Red-Green-Blue images instead of 16 bit images (gold lut for instance) reduced the depth of information and thus apparently reduces differences in densities within spines. Thus, in the revised version of the manuscript we used 16 bit images for PALM or dSTORM images alone but kept the 8 bit Red-Green-Blue images to illustrate the co-localization between proteins.

The partial overlapping of ArpC5A with the PSD and the fact that ArpC5A never concentrated at protrusion tips, along with the localization of WAVE subunits and IRSp53, argue for the stimulation of Arp2/3 nucleation close to the PSD (see the following answer).

The sequence of molecular events leading to the formation of an F-actin branch is still not completely understood. As pointed out by the reviewer, the localization of immobilized/confined Arp2/3 complex is not equivalent to the localization of F-actin branches. This was clearly demonstrated in recent *in vitro* experiments using purified Arp2/3 (references from Gelles laboratory: Smith *et al*., PNAS 2013; Smith *et al*., eLIFE 2013). In those experiments, the authors demonstrated that only a few percentages of Arp2/3 complexes binding to mother actin filaments will lead to the formation of F-actin branches (~ 2%). The remaining ~ 98% will bind to mother actin filaments with time constants of 2s (~90% of events) or 10–15 s (~8% of events). In our experiments, we are imaging Arp2/3 complexes associated with F-actin, but we cannot differentiate Arp2/3 complexes at the origin of an actin branch from Arp2/3 complexes associated with F-actin without the subsequent formation of a branch.

Nevertheless, the formation of F-actin branches is stimulated by Nucleating Promoting Factors, including the WAVE complex. Our data clearly show that in dendritic spines, WAVE complex subunits are concentrated close to the PSD. Thus, we have indirect evidence that stimulation of F-actin branches is mainly occurring close to the PSD. This is consistent with the co-localization, although partial, of ArpC5A and PSD-95 (note that Abi1 and Nap1 are strongly overlapping with PSD-95). Thus, from our results we can hypothesize that most Arp2/3-mediated nucleation events stimulated by the WAVE complex occur close to the PSD.

The localization of Arp2/3 complexes outside the PSD is not contradictory with this model. Indeed, there at least two ways to explain this localization:

1. Since the Arp2/3 complex could bind to F-actin for 10-15 seconds independently from its binding to NPF (references from Gelles laboratory: Smith *et al*., PNAS 2013), Arp2/3 could be associated with F-actin networks away from the PSD. However, in this case this will not lead to the formation of an actin branch and thus will not lead to further F-actin elongation. Note that the dynamics of Arp2/3 in spines is fast, as demonstrated by our sptPALM experiments (Supplementary Fig S9A; mEOS2-Arp2/3 are immobilized in spine for less than 30 s).
2. The formation of an F-actin branch also requires NPF dissociation from Arp2/3 (Smith *et al*., eLIFE 2013). Thus, once an actin branch has been initiated by the WAVE complex close to the PSD the Arp2/3 complex at its origin could be displaced away from the PSD by the forces generated by the growth of the F-actin network. This mechanism is consistent with *in vitro* reconstituted system demonstrating the delocalization of the Arp2/3 complex from a nucleating surface composed of VCA domains (Blanchoin Laboratory: Galland *et al*., Nat Materials 2013).

In the revised manuscript, we took special attention to remove all sentences that use the location of ArpC5A alone as an argument for nucleation close to the PSD. We mainly use the localization of WAVE complex subunits close to the PSD as a stronger argument for the preferred activation of the Arp2/3 complex close to the PSD. And as stated above, we are now referring to the proteins (nucleation promoting factors (NPFs) and elongation factors) and not to the functions (nucleation and elongation).

- the part on Rac1 in spines seems rather shaky and fuzzy. A lot of spinning around the fact that Rac1 is obviously freely diffusing and can therefore bind to the WAVE-complex. So what? Am I missing here anything that is beyond obvious and expected observation ?

Our results suggest that Rac1 activation is correlated with its immobilization since the fraction of immobilization is higher for Rac1-Q61L than for Rac1-WT or Rac1-T17N. Those results are in agreement with a recent study performed on adhesion sites of motile cells (Shibata *et al*., Cytoskeleton 2013). What is puzzling to us is that wild type mEOS2-Rac1 is as freely diffusing as its prenylated CAAX box. The low fraction of immobilization and confinement and fast free-diffusion of mEOS2-Rac1 in spines compared to WAVE complex subunits (Abi1 and Nap1) and IRSp53 is surprising given their direct interactions (Suetsugu *et al*, 2006). As we explain in the Results and Discussion sections, we think that the large fraction and fast free diffusion of mEOS2-Rac1 suggest that Rac1 binding to its targets, including the WAVE complex in spines, is faster than our acquisition frequency. This hypothesis is consistent with the low binding affinity between the

WAVE complex and Rac GTPase (Koronakis *et al*, 2011). If binding events are lasting only for a few ms, our acquisition frequency of 50 ms we will not be sufficient to temporally resolve them.

In the revised version we tried to simplify this part of the manuscript:

- Page 11 we replace ‘Since the WAVE subunits and IRSp53 displayed confinement and immobilization close to the PSD, Rac1 interactions with those proteins (Suetsugu *et al*, 2006) could trigger Rac1 confinement and/or immobilization.’ by ‘The higher fraction of free-diffusion and faster free-diffusion of mEOS2-Rac1 in spines compared to the WAVE complex subunits and IRSp53 is surprising given their direct interactions (Suetsugu *et al*, 2006).’.

- Page 15 we added ‘The large fraction of fast membrane free diffusion for Rac1 wild type compared to Abi1 and Nap1 suggests that Rac1 interact only transiently with the WAVE complex, consistent with their low binding affinity (Koronakis *et al*, 2011; Chen *et al*, 2010).’.

- the last chapter on dendritic spine enlargement is the weakest part. The authors are trying to explain finding upon overexpression of constitutively active Rac-mutants, which leads to enlarged spine structures. In the end the authors take this as an indication that enhanced synaptic activity might trigger WAVE delocalization and Arp2/3 activation. This is misleading. The neurons are transfected and then cultured for many days and at the end when the experiments are performed we are looking at neurons which have adapted for 10 days or so to constitutively active Rac1. This has nothing to do with 'stimulus triggered spine enlargement'. If they want to add anything here the authors must look at acute treatment of neurons (e.g.BDNF?!) and then look at the actin machinery.

After knowing the nanoscale organization and dynamics of F-actin regulators in resting conditions, the logical step is to study how decreased or increased synaptic activity regulates this dynamic organization. Thus, since the beginning of our project we wanted to vary synaptic activity to trigger changes in the morphology of spines.

As suggested by all the reviewers, inducing spine enlargement with an acute treatment would be more relevant when talking about long term potentiation (LTP). Although 24h bath application of BDNF was shown to induce an increase in spine width and length in hippocampal cultured neurons (Ji *et al*., 2010, Nature Neuroscience), 20min incubation, either in hippocampal cultured neurons and in hippocampal slice cultures, doesn’t induce an increase in spine volume (Hu *et al*., 2011, The Journal of Neuroscience; Tanaka *et al*., 2008, Science). So as suggested by reviewers 2 and 3, and before the submission of the manuscript, we used chemical LTP (cLTP) protocols to trigger synaptic plasticity in dissociated hippocampal neurons (Lu et *al*., 2001 Neuron; Park *et al*., 2004 Science; Wang *et al*., 2008 Neuron). These protocols, using Glycine stimulation of NMDA receptors, are known to induce increased frequency and amplitude of mEPSCs and a rapid insertion of AMPA receptors in spines (Lu *et al*., 2001 Neuron).

We used 200 µM glycine, 5 µM picrotoxin to induce cLTP in our dissociated hippocampal neurons (Lu *et al*., 2001 Neuron; Park *et al*., 2004 Science; Wang *et al*., 2008 Neuron). This stimulation protocol (3-5 mins) was performed in the presence of extracellular Mg2+. The same protocol performed in our institute with the same source of hippocampal neurons is inducing an increase in the amount of AMPA receptors (GluA1) in spines (Dupuis *et al*., EMBO J 2014). Thus, this protocol was efficient in triggering functional synaptic plasticity. However, quantification of spine size before and after this cLTP protocol, using EGFP as a reporter of spine volume, revealed no significant changes of the size of spines (see attached Referee Figure 1).

Because structural plasticity was not induced in our experimental conditions, we decided to change the morphology of spines by affecting actin regulators directly (over expression of constitutively active Rac1 (Tashiro and Yuste, Mol Cell Neurosci 2004) and over-expression of Shank3 (Durand *et al*., Mol Psy 2012; Han et al., Nature 2013)). We completely agree with the reviewers that this protocol is not equivalent to 'stimulus triggered spine enlargement' and we will discuss this point later. Nevertheless, this strategy allowed us to show that the nanoscale organization and dynamics of F-actin regulators in spines we found in basal conditions could be regulated.

Recently, two studies reported spine structural plasticity in dissociated neurons after cLTP (Fortin *et al*., J Neurosci 2010; Bosch *et al*., Neuron 2014). To address the reviewers’ comments, we decided to perform additional experiments using the cLTP protocol described in those studies. The major differences compared to the protocol we used previously is the absence of Mg2+ during stimulation

and longer stimulation (10 min versus 3-5 min), which induce stronger activation of NMDA receptors. This protocol successfully induced spine enlargement in hippocampal neurons as described in the studies of Fortin 2010 and Bosch 2014. We used the same protocol on our dissociated hippocampal neurons and used two different reporters of spine size (actin-GFP (Fortin 2010) and GFP (Bosch 2014)). Again, quantifications revealed no changes in the size of spines before versus after the cLTP protocol (see attached Referee Figure 1). To test if this protocol was nevertheless inducing functional synaptic plasticity, we verified the increased insertion of GluA1 in spines. Using immuno-labelling of endogenous GluA1, we showed that the amount of GluA1 was significantly increased after the same cLTP protocol (see attached Referee Figure 1).

One possibility to explain the lack of structural LTP after cLTP protocols in our dissociated neurons is that the majority of spines already reached a size close to their maximal size. This lack of stable enlargement was also described for large spines in hippocampal slices after Glutamate uncaging (Matsuzaki *et al.,* Nature 2004).

In any case, the best way to study structural LTP will be in hippocampal slices using glutamate uncaging (Murakoshi *et al*., Nature 2011 (Yasuda's lab); Matsuzaki *et al*., Nature 2004 (Kasai's lab); Bosch *et al*., Neuron 2014 (Hayashi's lab)). The major difficulty is then to couple slice imaging with (spt)PALM or (d)STORM. This is not trivial and will require a lot of adjustments which are beyond the timeline of this revised manuscript. However, this is exactly the direction we are following to address the links between structural plasticity and transient reorganization of F-actin regulators.

Given the fact that structural plasticity induced by cLTP is not efficient in our experimental conditions we would like to use our previous strategy (over expression of constitutively active Rac1 and over-expression of Shank3) to support a model where changes in the morphology of spines are associated with nanoscale reorganization of F-actin regulators. We think that being able to challenge the nanoscale organization of F-actin regulators (using Rac1 and Shank3) is a valuable result suggesting that the nanoscale dynamic organization of F-actin regulators could be modified and that this could be at play during morphological remodeling of spines.

However, in this new version of the manuscript, we tune down possible misleading statement or conclusions that might be interpreted as LTP or synaptic plasticity being demonstrated to be triggered by reorganization of F-actin regulators. In the manuscript, we paid special attention to remove strong statements concerning our results and synaptic plasticity. We rather refer to morphological remodeling of spines in general, that could be involved during transient (synaptic plasticity) and long lasting (pathophysiological) changes of spine morphology. Specifically:

- In the abstract we changed 'Thus the specific localization of branched F-actin regulators in spines might be reorganized during changes in synaptic activity to regulate spine shape' to 'Thus, the specific localization of branched F-actin regulators in spines might be reorganized during spine morphological remodeling often associated with synaptic plasticity.'
- Page 12 we replaced 'Spine enlargement during structural plasticity is triggered by changes in F-actin polymerization (Okamoto *et al*, 2004) and may thus be associated with reorganization of F-actin regulators within spines (Park *et al*, 2012).' by 'Spine morphological remodeling is triggered by changes in F-actin polymerization (Okamoto *et al*, 2004) and may thus be associated with reorganization of F-actin regulators within spines (Park *et al*, 2012; Bosch *et al*, 2014)'.
- Page 12 we replaced 'However, upon enhanced synaptic activity, delocalization of WAVE could be the prerequisite to trigger Arp2/3 activation and F-actin elongation throughout the spine and thus to induce its enlargement.' by 'However, transient or long lasting delocalization of the WAVE complex from the PSD could promote Arp2/3 activation and F-actin elongation throughout the spine, thereby triggering morphological remodeling.'.
- Page 13 we replaced 'Thus, controlling the spatiotemporal association between PSD components and F-actin regulators might be at the basis of actin remodeling during synaptic plasticity.' by 'Thus, control of the spatiotemporal association between PSD components and F-actin regulators might be at the basis of actin remodeling often

associated with synaptic plasticity and abnormal spine morphologies in neurological disorders.'.

- We completely modified the last part of the discussion that was previously only focused on synaptic plasticity. We now discuss acute (synaptic plasticity) and long lasting (pathophysiological) changes of spine morphology.

Referee #2:

The paper by Chazeau et al. used a state-of-art superresolution technique to elucidate the dynamics of multiple actin interacting proteins within dendritic spine. It has very unique information and experiments are generally well conducted. This reviewer trusts that it is highly appropriate for publication in EMBO J.

There are a few points need to be clarified.

Tatavarty et al. and Frost et al. used a very similar technique of single particle tracking and found a rearward movement of actin. Also, Honkura used PA-GFP actin and observed a similar, though it was bulk imaging. Where is this discrepancy coming from?

We thank the reviewer for those constructive questions and comments.

Our results are not questioning the existence of F-actin rearward movements, but the fact that these rearward movements are mainly and solely driven by F-actin growth against the tip of membrane protrusions, like is the case in the lamellipodium.

Indeed, like in the studies of Tatavarty *et al*., PLoS One 2009 and Frost *et al*., Neuron 2010, we also detected a fraction of F-actin moving rearward, but about the same fraction was moving forward. Thus, our results show that actin filaments and Arp2/3 complexes in spines are not undergoing a concerted rearward flow as opposed to the lamellipodium (Tatavarty *et al*., PLoS One 2009; Iwasa and Mullins Curr. Biol. 2007) and the growth cone (Medeiros *et al*., Nat Cell Biol 2006, our results).

The use of Cytochalasin D (Tatavarty, our study) or Jasplakinolide (Frost) to stop or decrease actin polymerization demonstrates that F-actin rearward movements depend on forces generated by actin polymerization, but do not demonstrate that these forces are triggered by the polymerization of F-actin against the tip of membrane protrusions. For example, forces could be exerted on a static (not polymerizing) capped actin filament by another actin filament that is polymerizing; likewise polymerization of an actin filament against the PSD will also generate movements; also forces generated by myosin motors on a stable actin filament will generate movements independently from actin polymerization.

In the lamellipodium several lines of evidence support the model where actin polymerization occurs mainly close to the protrusion tip pushing away the existing F-actin network:

1. The nucleating promoting factor activating Arp2/3 in the lamellipodium, the WAVE complex, is located at the tip of membrane protrusions along with F-actin elongators (VASP, FMNL2).
2. The fractions of forward F-actin movements and immobile F-actin are low (Tatavarty *et al*., PLoS One 2009; Iwasa and Mullins Curr. Biol. 2007).
3. FRAP/Photoactivation experiments clearly demonstrated that old actin filaments are completely replaced by new actin filaments (Lai *et al*., EMBO J 2008).
4. Rearward F-actin motions are generated independently from any membrane movements, i.e. the tip of the lamellipodium could be stationary and F-actin moving rearward (Giannone *et al*., Cell 2004; Ponti *et al*., Science 2004).
5. Finally, There is an inverse relationship between the rate of F-actin flow and membrane protrusion (Giannone *et al*., Cell 2004).

In dendritic spines, some of these conditions are not true or could not yet be proven experimentally:

1. The WAVE complex in spines is not localized at the tip of membrane protrusions but VASP and FMNL2 elongators are (our results).

2. The fractions of forward F-actin movements and immobile F-actin are high (Tatavarty *et al*., PLoS One 2009, our results).
3. Experiments showed that a significant fraction of F-actin photoactivated at the spine apex remained at its initial position tens of seconds after photoactivation (Honkura *et al*., Neuron 2008; Figure 2B), as opposed to similar experiments performed in the lamellipodium (Lai *et al*., EMBO J 2008; Figure 1B). This is consistent with the absence of a fast concerted F-actin flow.
4. Dendritic spines are constantly undergoing morphological changes since they constantly send and withdraw membrane protrusions (Fischer *et al*, Neuron 1998; Dunaevsky *et al*, PNAS 1999; Lendvai *et al*, Nature 2000; Berning *et al*, Science 2012). Thus, F-actin backward movements might result from protrusions ruffling back to the spine center (Berning *et al*., Science 2012; associated movie).
5. It is currently not possible to simultaneously and with sufficient spatial and temporal resolution measure F-actin movements and membrane movements. Thus we cannot determine the relationship between the rate of F-actin flow and membrane protrusion.

For all those reasons, we wanted to point out in our manuscript that currently in the literature, there is no strong evidence that like in the lamellipodium, F-actin movements are driven by F-actin polymerization against the tip of membrane protrusions.

Note that we are not questioning the net slow bulk rearward F-actin flow demonstrated using photoactivation experiments (Honkura *et al*., Neuron 2008; Frost *et al*., Neuron 2010). But again there is no formal prove that this slow flow is mainly and solely driven by F-actin polymerization against the tip of membrane protrusions:

1. This net flow could be due to the geometry of the system composed of the dendritic spine plus the entire shaft. The progressive loss of F-actin filaments from the spine apex could be due to the fact that actin filament are sunk from the spine into the dendritic shaft.
2. This slow flow might result from F-actin severed from the apex of protrusions that could be recycled at the spine center to prime new nucleation events (Achard *et al*., Curr. Biol. 2010). This could be consistent with the slow redistribution of F-actin filaments observed after photoactivation at the spine apex (Honkura *et al*., Neuron 2008).
3. Finally, slow forward redistribution of F-actin was also demonstrated in dendritic spines especially during LTP protocol (Honkura *et al*., Neuron 2008; Figure 2C and 5D). These forward redistributions support our model where branched F-actin networks are nucleated at or close to the PSD, providing barbed-ends that are then elongated outwards from the PSD.

We removed in the manuscript all the sentences stating that no rearward flow of F-actin is occurring in spines:

- Page 31 we replaced the title of Figure 1 ‘Arp2/3 complex and associated F-actin branches are not moving rearward in spines’ by ‘Arp2/3 complex and F-actin movements are not polarized in spines’.

Experimentally, the differences between our study, the studies of Tatavarty *et al*., PLoS One 2009 and Frost *et al*., Neuron 2010, are small. In our study and in the study of Tatavarty, we applied a threshold for the length of trajectories analysed to increase the accuracy of speed measurement, respectively >6 points (12 s at 0.5 Hz) versus >8 points (4s at 2 Hz). However, in the study of Frost *et al.*, they included all the trajectories (2 points and over). They subdivided F-actin velocities in 3 time intervals: 38.8 nm/s for the first 2s, 13.5 nm/s between 2s and 8s, and 0.9 nm/s between 8 and 14s. Thus, besides the measurements performed at short times in the Frost study (which are faster), the velocity of F-actin measured in the 3 studies are similar. In addition, both studies also demonstrated the existence of forward directed motions. Although the proportion of forward flow is not quantified in the study of Frost *et al*., Neuron 2010, it is clearly visible in figure 5 and discussed in the manuscript. Indeed, the authors point out that the bulk rearward flow observed using conventional fluorescent microscopy (Honkura and their results) might obscure local flow not directed form the tip to the base of spines:

‘Using targeted photoactivation, we observed as reported previously (Honkura et al., 2008) that spines possess a general tip-to-base orientation of actin flow. However, both an optical monomer-incorporation assay and single-molecule tracking revealed that this does not result strictly from preferential polymerization activity at the spine tip or at the synapse. Rather, sites of high polymerization activity are broadly distributed and found at spine tips, in lateral domains, and even

in or near the neck. These distributed sites likely represent points of regulated control over filament density, length, and turnover. Thus, the tip-to-base flow structure that can be resolved via relatively low resolution confocal microscopy appears to be an emergent phenomenon that masks a more intricate and functionally revealing underlying organization.'

In the original study of Tatavarty *et al*., PLoS One 2009, they found 10 % anterograde, 34 % retrograde movements and 37 % of stationary actin filaments. These results suggest, similarly to our results, that there is no concerted flow of actin in spines as opposed to the lamellipodium (Lai *et al*., EMBO J 2008; Iwasa and Mullins Curr. Biol. 2007) or the growth cone (Medeiros *et al*., Nat Cell Biol 2006, our results). Their conclusion from these values was:

'On the other hand, the existence of many randomly moving molecules cannot be accounted for by the first mechanism (which is the mechanism of actin flow in the lamellipodium: actin polymerization pushing against the membrane). It is likely that the second mechanism plays an important role in spines (polymerization of randomly oriented short actin filament).'

In conclusion, we think that there are no major discrepancies between our study and the study of Tatavarty and Frost. The major discrepancy is rather the interpretation of the results. We are not convinced that F-actin rearward movements in spines are mainly and solely driven by F-actin growth against the tip of membrane protrusions, like it is the case in the lamellipodium.

Finger-like extensions the authors observe resembles what other people call "spinules". The identity of these two structures (or otherwise) should be carefully tested. Also, EM reconstruction studies of spines in vivo such as those by Harris seldom see such structure. So the frequent occurrence of the finger-like extension may be some artifact unique to the author's system.

To test if 'spinules' and the finger-like extensions we observed are the same or distinct structures will require to perform correlative Electron Microscopy and super-resolution fluorescence microscopy. Although some groups are able to perform such type of experiments (Watanabe *et al.,* Nat Meth 2011 (Jorgensen's lab); Kopek *et al.,* PNAS 2012 (Hess's lab)) it is currently beyond our capabilities.

Several line of evidences suggest that 'Spinules' observed using EM might correspond to actin-dependent membrane protrusions observed using conventional fluorescent microscopy:

1. Spinules, as defined using EM, are small narrow projections usually originating from the head or neck of a spine (Sorra *et al.,* J of Comparative Neurology 1998). Thus, 'Spinules' observed using EM could correspond to F-actin membrane protrusions observed using conventional fluorescent microscopy (Edwards, Nature 1998; Izeddin *et al*, Plos One 2011; Fischer *et al*, Neuron 1998). In agreement with this hypothesis, the formation of Spinules, as defined in a recent paper from Hayashi's laboratory, are actin-dependent protrusions leading to spine enlargement triggered by structural LTP of spines (Ueda and Hayashi, J. Neuroscience 2013). Importantly, the Spinules as defined in this study look very similar to membrane protrusions observed using conventional fluorescent microscopy in other seminal studies (Fischer *et al.,* Neuron 1998; Dunaevsky *et al.,* PNAS 1999; Lendvai *et al.,* Nature 2000). Another study using fluorescent microscopy also described the formation of spine head membrane protrusions upon glutamate stimulation (Richards *et al.,* PNAS 2005). Thus 'Spinules' in classical fluorescence microscopy probably correspond to membrane protrusions.
2. Many studies demonstrated that spines are motile structures both in neuronal cultures, brain slices and in intact brains (Fischer et al., Neuron 1998; Dunaevsky *et al.,* PNAS 1999; Lendvai *et al.,* Nature 2000). Recent studies that used STED microscopy in acute brain slices revealed the existence of membrane protrusions extending from the spine head (Bethge et al., 2013, Biophysical Journal, Figure 4A (Nagerl's lab); Takasaki *et al.,* 2013 (Sabatini's lab), Biophysical Journal, Figure 5A, 7A (Sabatini's lab)). Furthermore, STED microscopy performed *in vivo* also showed that dendritic spines constantly send and retract membrane protrusions (Berning *et al.,* 2012, Science (Hell's lab)). Thus, these studies suggest that spine motility and perhaps the formation of 'Spinules' is not a rare event in dendritic spines.
3. In fact the formation of 'Spinules' and the formation of actin-dependent protrusions leading to spine enlargement are both triggered by enhanced synaptic activity. EM studies showed that the number of 'Spinules' increases with synaptic activation and plasticity (Schuster et al., Brain Research 1990; Toni *et al.,* Nature 1999, Tao-Cheng *et al.,* Neuroscience 2009).

Thus, these results support that the 'Spinules' observed by Ueda and Hayashi, are 'Spinules' observed in EM.

4. Concerning the occurrence of 'Spinules' in EM studies, the conclusions are not consistent. A study from the Harris's group (Spacek and Harris, J Neuro 2004) showed that 'Spinules' are present in 30 % of all spines. Importantly, 90 % of mushroom spines contained 'Spinules' (spines without 'Spinules' mostly correspond to thin spines). According to a study from Reese's laboratory, spines do not present any 'Spinules' at basal condition but formed in response to sustained synaptic activity (Tao-Cheng *et al.,* Neuroscience 2009).

All together, these results indicate that 'Spinules' might correspond to actin-dependent protrusions, which are often associated with enhanced synaptic activity.

Following are the evidences showing that the finger-like extensions we are observing using super-resolution microscopies (PALM, dSTORM) correspond to membrane extensions observed using classical fluorescence microscopy:

1. Finger-like extensions are membrane protrusions as demonstrated in Supplementary Figure S2.
2. Actin is localized in those protrusions and actin elongators (VASP and FMNL2) are concentrated at the tip of these finger-like protrusions strengthening the hypothesis that they form in response to F-actin elongation (Figure 2 and Supplementary Figure S1).
3. Stopping actin polymerization using Cytochalasin D (CD) leads to inhibition of spine motility (Fischer *et al.,* Neuron 1998; Dunaevsky *et al.,* PNAS 1999). We performed additional PALM experiments on neurons transfected with mEOS2-VASP. Those experiments showed that CD treatment disrupted the formation of finger-like protrusions. We added these results in Supplementary Figure S2, and we replaced in the results section (page 6) 'Time-lapses demonstrated that those extensions corresponded to protrusions moving forwards with velocities close to the ones measured for lamellipodia and filopodia (~60 nm/s; 22 protrusions) (Svitkina *et al*, 2003; Giannone *et al*, 2004) (Supplementary Fig S2).' By 'Time-lapse recordings demonstrated that those extensions corresponded to protrusions, lost after CD treatment, and moving forwards with velocities close to the ones measured for lamellipodia and filopodia (~60 nm/s; 22 protrusions) (Svitkina *et al*, 2003; Giannone *et al*, 2004) (Supplementary Fig S2).'.

Therefore 'Spinules' (EM), finger-like extensions (PALM, dSTORM) and membrane protrusions (classical fluorescence microscopy) might correspond to the same structure. But again, the definitive prove will require correlative Electron Microscopy and super-resolution fluorescence microscopy. Note that in our manuscript we are not stating that the protrusive structures we observed correspond to 'Spinules'.

The discrepancy between the low occurrence of 'Spinules' in EM (but keep in mind the fact that 90% of mushroom spines bear 'Spinules') and the fact that we observe 70% of spines with finger-like protrusions might be related to differences in the basal activity of neurons in the different experimental conditions. As pointed-out before, the formation of 'Spinules' is associated with enhanced synaptic activity, as the formation of membrane protrusions were observed using classical fluorescence microscopy (Ueda and Hayashi, J. Neuroscience 2013, Richards *et al.,* PNAS 2005). Thus, the fact that we observed finger-like protrusions in 70% of spines might be due to an enhanced neuronal activity in our experimental conditions. This could also explain why chemical LTP protocols are not inducing an increase in the size of spines in our dissociated neurons (see 'Other comments #5').

To clarify this part of the manuscript and add important references supporting that 'spinules' and membranes protrusions responsible for spine motility might be the same structures, we replaced page 13: 'However, EM micrographs also demonstrated the existence of thin actin-dependent protrusions emerging from the spine head named spinules (Weiler & Janssen-Bienhold, 1993) that could correspond to F-actin membrane protrusions (Edwards, 1998; Izeddin *et al*, 2011; Fischer *et al*, 1998; Berning *et al*, 2012).' by 'However, EM micrographs also demonstrated the existence of thin protrusions emerging from the spine head named spinules (Spacek & Harris, 2004) that could correspond to F-actin membrane protrusions (Edwards, 1998; Izeddin *et al*, 2011; Fischer *et al*, 1998; Berning *et al*, 2012; Ueda & Hayashi, 2013).'.

Other comments.

1. Spine/protrusion of mEos2-VASP expressing neuron looks abnormal (Fig.2B). Molecular behavior in abnormal neuron would not be normal. Similarly, mEos2-Rac1-T17N expressing neuron also showed abnormal morphology (Fig. 6E).

Overall the spines of neurons transfected with mEOS2-VASP look normal (see Fig 3A and Supplementary Fig S2A). In addition, mEOS2-VASP over-expression do not affect the size of the PSD, like in the majority of our conditions, with the exception of mEOS2-IRSp53 (see Supplementary Fig S6). Likewise, over-expression of FMNL2-mEOS2 does not drastically affect the morphology of spines (see Fig 2C and Supplementary Fig S2B). Nevertheless, both transfected and endogenous VASP and FMNL2 are concentrated at the tip of membranes protrusions (above mentioned figures plus Supplementary Fig S1) demonstrating that F-actin elongations are mainly occurring at the tip of finger-like protrusions in spines.

Therefore, although over-expression of VASP could sometimes induce spines with altered morphologies, this do not change the main message of those experiments which is the localization of F-actin elongators at protrusion tips in spines.

Concerning mEos2-Rac1-T17N over expression, as demonstrated by others (Tashiro and Yuste, Mol Cell Neurosci 2004), over-expression of dominent negative Rac1 induced the transformation of spines into non-motile filopodia-like structures. Importantly, Latrunculin A (LatA) treatment, which induced loss of actin-GFP and GFP-ArpC5A accumulation in spines (Supplementary Fig S7), also transformed spines into non-motile filopodia-like structures (Figure 6E and Supplementary Fig S8). Alltogether, these results strongly suggest that the continual nucleation of branched F-actin networks maintains the globular shape of the spine head.

As for the molecular behavior in neurons exhibiting 'abnormal' morphologies, e.g. mEos2-Rac1-T17N over-expressing neurons, this is discussed in the 'Other comments #4'. We discuss the fact that the diffusive behavior of a protein is not completely determined by the morphology of spines.

2. Fig. 3A and C. In the provided images, it is hard to tell whether the dot like structure exist at the tip of the singer-like extension or within spine. As a matter of fact, dot-like signals are all over. Also, what it the structure extending upward from in Fig 3A green channel?

There is a major difference between live (including Fig 2 and Supplementary Fig S2, S3) and fixed experiments (including Fig 3). In live experiments, fast free-diffusing protein fused to mEOS2 (membrane or cytosolic proteins) will outline the spine shape while confined and immobile mEOS2 will accumulate in sub-spine domains; the central domain for WAVE complex subunits, the protrusion tips for VASP/FMNL2, and Arp2/3 nano-domains. However, in fixed experiments (dSTORM or dSTROM/PALM), the fast free-diffusing proteins will be immobilized, due to fixation, in locations that do not correspond to their real accumulation in specific sub-spine domain. Thus, in fixed experiments, those 'false immobile' proteins add noise to the 'real confined/immobilized' proteins selectively observed in live experiments. Those 'non-specific' immobilization contribute to the dot-like signals more pronounced in fixed experiments.

The live experiments allowed us to clearly differentiate the localization of mEOS2-fused proteins. Our data demonstrated that subunits of the WAVE complex (Abi1 and Nap1) and IRSp53 form a central domain co-localized with the PSD, while VASP and FMNL2 move outwards from the PSD with protrusion tips. These conclusions are supported by Figure 2, 3 and Supplementary Figure S3. Compare Figure 2B, C (VASP/FMNL2) with Supplementary Figure S3 (Abi1 and Nap1) and Supplementary Figure S6 (IRSp53).

There was also a problem of representation in PALM/dSTORM experiments (Fig 3, 7, S5, S6 and S10). Using 8 bit Red-Green-Blue images instead of 16 bit images (gold lut for instance) reduced the depth of information and thus apparently reduces differences in densities within spines. Thus, in the revised version of the manuscript, we used 16 bit images for PALM or dSTORM images alone but kept the 8 bit Red-Green-Blue images to illustrate the co-localization between proteins.

Like demonstrated in the laboratory of Yuste, dendritic spines exhibit different types of morphological rearrangements. Sometimes, large membrane protrusions are transiently extending from dendritic spines (see Dunaevsky *et al.,* PNAS 1999 Fig. 2e). The structure in Fig. 3A could correspond to such a membrane protrusion. Note that spines that presented such large protrusions were not analyzed to generate the Fig. 3D and E.

3. Fig. 4H. It is a little surprising that cytosolic-mEOS2 shows a significant immobile fraction. Could it be an error of tracking? Protein aggregation? Has the authors tried mEOS3?

A protein freely diffusing in the cytosol is characterized by coefficient of diffusion (D) around 100 times faster than a protein freely diffusing in the membrane. With our acquisition parameters and with the limit of our tracking analysis, we are not able to reconnect trajectories of proteins freely diffusing in the cytosol with very fast D. In addition, we are using oblique (parallel) illumination which limits further the probability to reconnect trajectories diffusing in 3D in the cytosol. Thus with our method we are not able to measure the fastest D for a protein freely diffusing in the cytosol.

However, our method and experimental conditions allow us to track easily transmembrane proteins or proteins associated with membrane components with D ranging from ~0.01µm^2/s to ~1µm^2/s.

In the case of a mEOS2 alone, the trajectories we can reconstruct correspond mainly to mEOS2 nonspecifically interacting with membrane components, leading to rare free diffusing and immobilization events on the membrane. Therefore, counter-intuitively, a mEOS2 alone freely diffusing in the cytosol will result in a distribution of D further shifted towards slower D compared to a protein freely diffusing on the membrane, compare Figure 4G-J (cyto, purple) and Figure 5G-I (CAAX, magenta).

However, we are be able to record trajectories of a cytosolic protein (fused to mEOS2) that will be transiently freely diffusing on the membrane if this cytosolic protein is interacting with membrane components that are freely diffusing (for instance, the WAVE complex Fig 5A). Similarly, we will be able the reconnect the trajectories of a cytosolic protein if this protein is associated with a 'static structure' relative to the frequency of acquisition we are using (for instance Arp2/3 interacting with F-actin, or actin incorporated into F-actin, Fig 4).

This bias of single protein tracking experiments is also true in cells having simpler geometry such as fibroblasts. In one of our previous studies (Rossier *et al.,* Nature Cell Biology 2012) the cytosolic protein talin (full length) presents apparently slower free-diffusing events than a transmembrane protein such as integrins.

To clarify this point, we added a paragraph in the Method section on page 19.

4. It would be better to show the data using Rac inhibitors to conclude Rac motility, because Rac-N17 expressing neuron has no mushroom spine (Fig.6E). Expecting result would be that inhibition Rac-GTP by EHT1864 increases free diffusion fraction of Rac1.

The use of the Rac1 inhibitor EHT1864 is a good suggestion. And we should probably use it to test the effects of an acute inhibition of Rac1 on the nanoscale dynamics and organization of Rac1 but also on downstream actin regulators.

Nevertheless, we think that the large fraction of fast free-diffusion of mEOS2-Rac1-T17N is not determined by the loss of spine heads. First, the diffusive behavior of mEOS2-Rac1-WT is similar to the behavior of mEOS2-Rac1-T17N, however spine morphologies are distinct. This suggests that the morphology of the spine, in this case at least, does not completely determine the diffusive behavior of proteins. Second, the diffusive behaviors of Rac1-WT and Rac1-T17N are also similar in dendritic shafts, which have the same morphology in both conditions. Finally, our results are in agreement with a recent study performed inside and outside adhesion sites of motile cells (Shibata *et al.,* Cytoskeleton 2013). Thus, also in cells having simpler morphology there are only slight differences in the diffusive behavior of Rac1-WT compared to Rac1-T17N.

5. In spine enlargement experiment, they used over expression of active Rac or Shank3 (Fig.7). But as it is well established that chemical-LTP protocol induces spine enlargement in dissociated neuron (Fortin et al., 2010; Bosch et al., 2014), chem.LTP would be more physiologically relevant system than over-expression of signaling molecules. Authors can compare molecular motility in a same spine at basal or potentiated condition.

After knowing the nanoscale organization and dynamics of F-actin regulators in resting conditions, the logical step is to study how decreased or increased synaptic activity regulates this dynamic organization. Thus, since the beginning of our project we wanted to vary synaptic activity to trigger changes in the morphology of spines.

As suggested by all the reviewers, inducing spine enlargement with an acute treatment would be more relevant when talking about long term potentiation (LTP). So as suggested by reviewers 2 and 3, and before the submission of the manuscript, we used chemical LTP (cLTP) protocols to trigger synaptic plasticity in dissociated hippocampal neurons (Lu *et al.,* 2001 Neuron; Park *et al.,* 2004 Science; Wang *et al.,* 2008 Neuron). These protocols, using Glycine stimulation of NMDA

receptors, are known to induce increased frequency and amplitude of mEPSCs and a rapid insertion of AMPA receptors in spines (Lu *et al.,* 2001 Neuron).

We used 200 µM glycine, 5 µM picrotoxin to induce cLTP in our dissociated hippocampal neurons (Lu *et al*., 2001 Neuron; Park *et al*., 2004 Science; Wang *et al*., 2008 Neuron). This stimulation protocol (3-5 mins) was performed in the presence of extracellular Mg2+. The same protocol performed in our institute with the same source of hippocampal neurons is inducing an increase in the amount of AMPA receptors (GluA1) in spines (Dupuis *et al*., EMBO J 2014). Thus, this protocol was efficient in triggering functional synaptic plasticity. However, quantification of spine size before and after this cLTP protocol, using EGFP as a reporter of spine volume, revealed no significant changes of the size of spines (see attached Referee Figure 1).

Because structural plasticity was not induced in our experimental conditions, we decided to change the morphology of spines by affecting actin regulators directly (over expression of constitutively active Rac1 (Tashiro and Yuste, Mol Cell Neurosci 2004) and over-expression of Shank3 (Durand *et al.,* Mol Psy 2012; Han *et al.,* Nature 2013)). We completely agree with the reviewers that this protocol is not equivalent to 'stimulus triggered spine enlargement' and we will discuss this point later. Nevertheless, this strategy allowed us to show that the nanoscale organization and dynamics of F-actin regulators in spines we found in basal conditions could be regulated.

Recently, two studies reported spine structural plasticity in dissociated neurons after cLTP (Fortin *et al*., J Neurosci 2010; Bosch *et al*., Neuron 2014). To address the reviewers' comments, we decided to perform additional experiments using the cLTP protocol described in those studies. The major differences compared to the protocol we used previously is the absence of Mg2+ during stimulation and longer stimulation (10 min versus 3-5 min), which induce stronger activation of NMDA receptors. This protocol successfully induced spine enlargement in hippocampal neurons as described in the studies of Fortin 2010 and Bosch 2014. We used the same protocol on our dissociated hippocampal neurons and used two different reporters of spine size (actin-GFP (Fortin 2010) and GFP (Bosch 2014)). Again, quantifications revealed no changes in the size of spines before versus after the cLTP protocol (see attached Referee Figure 1). To test if this protocol was nevertheless inducing functional synaptic plasticity, we verified the increased insertion of GluA1 in spines. Using immuno-labelling of endogenous GluA1, we showed that the amount of GluA1 was significantly increased after the same cLTP protocol (see attached Referee Figure 1).

One possibility to explain the lack of structural LTP after cLTP protocols in our dissociated neurons is that the majority of spines already reached a size close to their maximal size. This lack of stable enlargement was also described for large spines in hippocampal slices after Glutamate uncaging (Matsuzaki *et al.,* Nature 2004).

In any case, the best way to study structural LTP will be in hippocampal slices using glutamate uncaging (Murakoshi *et al.,* Nature 2011 (Yasuda's lab); Matsuzaki *et al.,* Nature 2004 (Kasai's lab); Bosch *et al.,* Neuron 2014 (Hayashi's lab)). The major difficulty is then to couple slice imaging with (spt)PALM or (d)STORM. This is not trivial and will require a lot of adjustments which are beyond the timeline of this revised manuscript. However, this is exactly the direction we are following to address the links between structural plasticity and transient reorganization of F-actin regulators.

Given the fact that structural plasticity induced by cLTP is not efficient in our experimental conditions we would like to use our previous strategy (over expression of constitutively active Rac1 and over-expression of Shank3) to support a model where changes in the morphology of spines are associated with nanoscale reorganization of F-actin regulators. We think that being able to challenge the nanoscale organization of F-actin regulators (using Rac1 and Shank3) is a valuable result suggesting that the nanoscale dynamic organization of F-actin regulators could be modified and that this could be at play during morphological remodeling of spines.

However, in this new version of the manuscript, we tune down possible misleading statement or conclusions that might be interpreted as LTP or synaptic plasticity being demonstrated to be triggered by reorganization of F-actin regulators. In the manuscript, we paid special attention to

remove strong statements concerning our results and synaptic plasticity. We rather refer to morphological remodeling of spines in general, that could be involved during transient (synaptic plasticity) and long lasting (pathophysiological) changes of spine morphology. Specifically:

- In the abstract we changed 'Thus the specific localization of branched F-actin regulators in spines might be reorganized during changes in synaptic activity to regulate spine shape' to 'Thus, the specific localization of branched F-actin regulators in spines might be reorganized during spine morphological remodeling often associated with synaptic plasticity.'
- Page 12 we replaced 'Spine enlargement during structural plasticity is triggered by changes in F-actin polymerization (Okamoto *et al*, 2004) and may thus be associated with reorganization of F-actin regulators within spines (Park *et al*, 2012).' by 'Spine morphological remodeling is triggered by changes in F-actin polymerization (Okamoto *et al*, 2004) and may thus be associated with reorganization of F-actin regulators within spines (Park *et al*, 2012; Bosch *et al*, 2014)'.
- Page 12 we replaced 'However, upon enhanced synaptic activity, delocalization of WAVE could be the prerequisite to trigger Arp2/3 activation and F-actin elongation throughout the spine and thus to induce its enlargement.' by 'However, transient or long lasting delocalization of the WAVE complex from the PSD could promote Arp2/3 activation and F-actin elongation throughout the spine, thereby triggering morphological remodeling.'.
- Page 13 we replaced 'Thus, controlling the spatiotemporal association between PSD components and F-actin regulators might be at the basis of actin remodeling during synaptic plasticity.' by 'Thus, control of the spatiotemporal association between PSD components and F-actin regulators might be at the basis of actin remodeling often associated with synaptic plasticity and abnormal spine morphologies in neurological disorders.'.
- We completely modified the last part of the discussion that was previously only focused on synaptic plasticity. We now discuss acute (synaptic plasticity) and long lasting (pathophysiological) changes of spine morphology.

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings Chazeau et al. study advance our understanding on the actin dynamics in dendritic spines, how actin polymerization can be translated into proper spine head morphology. The main finding is that actin is nucleated next to PSD but polymerization of the filaments occurs at the tip of finger-like protrusions. Especially the idea that nucleation locates stably next to PSD is new. Model is well supported by the presented data. The idea that neuron activation induced changes are achieved through the WAVE mobilization from the PSD area is good but I don't think the experiments used here are really relevant to support this idea.

Technically manuscript seems to be solid, special thanks for presenting the normal resolution images next to super resolution images. Those images make it easier to see how dendrites look like for readers who are not used to super resolution images. They also nicely highlight how super resolution images differ from normal resolution images.

Specific major concerns essential to be addressed to support the conclusions

1) Spine enlargement model is interesting but the problem is that enlargement is induced here by over-expression of constitutively active Rac1 or over-expression of Shank3. It is possible that when these proteins are over-expressed they start to activate WAVE in "wrong" locations resulting for example lamellipodia on the dendrites, which is obviously not the normal situation. It is also unclear if these over-expressions have anything to do with neuron activation induced spine enlargement. Based on the recent article from Hayashi laboratory (Bosch et al., Neuron 2014), expression levels of PSD proteins, such as Homer1b and Shank1b, increases 60 minutes after the fast spine enlargement and it is plausible that in contrast to induce actin rearrangements, they stabilize the actin network. Therefore it is questionable what the over-expression experiments can really tell us. If authors want to test neuron activation induced spine head growth they should perform some neuron activation, easiest might be the chemical LTP. Although chemical LTP is not the most

fanciest method to activate neurons it would be anyway more relevant way to address this question than activating Rac1 or over-expressing Shank3.

We thank the reviewer for those constructive questions and comments.

After knowing the nanoscale organization and dynamics of F-actin regulators in resting conditions, the logical step is to study how decreased or increased synaptic activity regulates this dynamic organization. Thus, since the beginning of our project we wanted to vary synaptic activity to trigger changes in the morphology of spines.

As suggested by all the reviewers, inducing spine enlargement with an acute treatment would be more relevant when talking about long term potentiation (LTP). So as suggested by reviewers 2 and 3, and before the submission of the manuscript, we used chemical LTP (cLTP) protocols to trigger synaptic plasticity in dissociated hippocampal neurons (Lu *et al.,* 2001 Neuron; Park *et al.,* 2004 Science; Wang *et al.,* 2008 Neuron). These protocols, using Glycine stimulation of NMDA receptors, are known to induce increased frequency and amplitude of mEPSCs and a rapid insertion of AMPA receptors in spines (Lu *et al.,* 2001 Neuron).

We used 200 µM glycine, 5 µM picrotoxin to induce cLTP in our dissociated hippocampal neurons (Lu *et al*., 2001 Neuron; Park *et al*., 2004 Science; Wang *et al*., 2008 Neuron). This stimulation protocol (3-5 mins) was performed in the presence of extracellular Mg2+. The same protocol performed in our institute with the same source of hippocampal neurons is inducing an increase in the amount of AMPA receptors (GluA1) in spines (Dupuis *et al*., EMBO J 2014). Thus, this protocol was efficient in triggering functional synaptic plasticity. However, quantification of spine size before and after this cLTP protocol, using EGFP as a reporter of spine volume, revealed no significant changes of the size of spines (see attached Referee Figure 1).

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In any case, the best way to study structural LTP will be in hippocampal slices using glutamate uncaging (Murakoshi *et al.,* Nature 2011 (Yasuda's lab); Matsuzaki *et al.,* Nature 2004 (Kasai's lab); Bosch *et al.,* Neuron 2014 (Hayashi's lab)). The major difficulty is then to couple slice imaging with (spt)PALM or (d)STORM. This is not trivial and will require a lot of adjustments which are beyond the timeline of this revised manuscript. However, this is exactly the direction we are

following to address the links between structural plasticity and transient reorganization of F-actin regulators.

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However, in this new version of the manuscript, we tune down possible misleading statement or conclusions that might be interpreted as LTP or synaptic plasticity being demonstrated to be triggered by reorganization of F-actin regulators. In the manuscript, we paid special attention to remove strong statements concerning our results and synaptic plasticity. We rather refer to morphological remodeling of spines in general, that could be involved during transient (synaptic plasticity) and long lasting (pathophysiological) changes of spine morphology. Specifically:

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- We completely modified the last part of the discussion that was previously only focused on synaptic plasticity. We now discuss acute (synaptic plasticity) and long lasting (pathophysiological) changes of spine morphology.

Minor concerns that should be addressed

1) "In the brain, most excitatory post-synapses are small membrane extensions called dendritic spines."?

I would not define dendritic spines as post-synapses. Dendritic spines are just protrusions where synapses locate.

Page 3 we replaced ‘In the brain, most excitatory post-synapses are small membrane extensions called dendritic spines.’ by ‘In the brain, most excitatory post-synapses are located in small membrane extensions called dendritic spines.’

2) Could authors explain the difference with the localization presented for WAVE complex in this manuscript and that presented for WAVE-1 in Soderling et al, J. Neuroscience 2007 (Fig. 2E)?

In the article of Soderling *et al.,* J. Neuroscience 2007 WAVE1-GFP expressed in neurons seems to fill the dendritic spines entirely (Fig. 2E). Without having access to the raw data, it is difficult to test if this high, homogenous concentration of WAVE1 in spines is linked to saturation of the fluorescent signal preventing to observe differences in intensity.

For the revised manuscript, to further support the nanoscale localization and dynamics of the WAVE complex in spines, we performed additional experiments on another subunit of the WAVE complex, Nap1.

Live super-resolution intensity images showed that mEOS2-Nap1 was not localized at protruding tips, but clustered into a single central domain within dendritic spines (see Supplementary Fig S3B). . Like mEOS2-Abi1, dual-color PALM/dSTORM experiments demonstrated that mEOS2-Nap1 detections formed a domain having the same size and overlapping with the PSD-95 domain (see Fig 3D, E and Supplementary Fig S5B). In addition, we performed high-frequency sptPALM acquisition (20 Hz) to characterize the diffusive properties of mEOS2-Nap1. Like mEOS2-Abi1, distributions of D for mEOS2-Nap1 were shifted towards faster diffusions compared to F-actin and Arp2/3 (see Supplementary Fig S5C-G). Both in shafts and spines, the fraction of immobilized mEOS2-Abi1 and mEOS2-Nap1 were lower, and the fractions of confined and free-diffusion were larger. Furthermore like mEOS2-Abi1, many confined and immobile mEOS2-Nap1 trajectories were detected in the same area (see Supplementary Fig S5D).

Thus, these results further support that the WAVE complex is retained in a confinement zone forming a long-lasting domain overlapping with the PSD.

Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

1) "Control of F-actin length and turnover in spines is then ensured by elongating factors such as formins (Hotulainen et al, 2009) and VASP (Lin et al, 2010), capping proteins (Fan et al, 2011; Korobova&Svitkina, 2010), and F-actin severing proteins such as ADF/cofilin (Gu et al, 2010; Rust et al, 2010; Bosch et al, 2014)."I understand how I should read this sentence but there is a possibility to misread it so that capping proteins and ADF/cofilins are also elongating factors. Maybe good to rephrase that.

We rephrased the sentence to:

Control of F-actin length in spines is then ensured by elongation factors such as VASP and formins (Hotulainen *et al*, 2009) (Lin *et al*, 2010) and by capping proteins (Fan *et al*, 2011; Korobova & Svitkina, 2010), while F-actin turnover depends on severing proteins such as ADF/cofilin (Gu *et al*, 2010; Rust *et al*, 2010; Bosch *et al*, 2014).

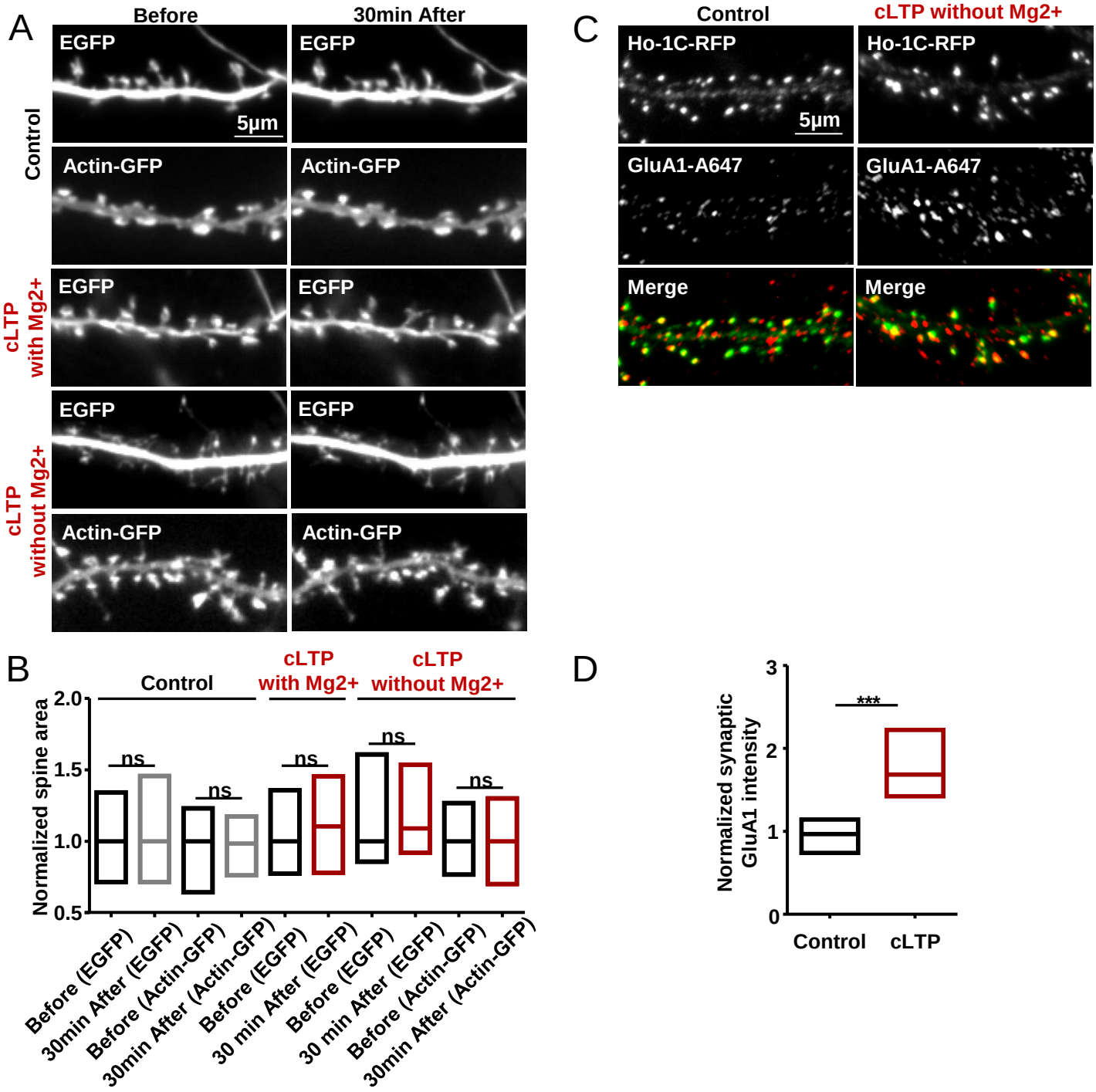
2) "Consequently, Arp2/3 and actin monomers are incorporated in branched F-actin networks growing inward from the lamellipodium tip." I would write that F-actin network GROW toward/against the tip of lamellipodium and FLOW inward.

We changed the sentence to:

'Consequently, Arp2/3 complexes and actin monomers are incorporated in branched F-actin networks growing against the lamellipodium tip, while ADF/cofilin associates with the entire network inducing F-actin severing (Iwasa & Mullins, 2007; Lai *et al*, 2008).'

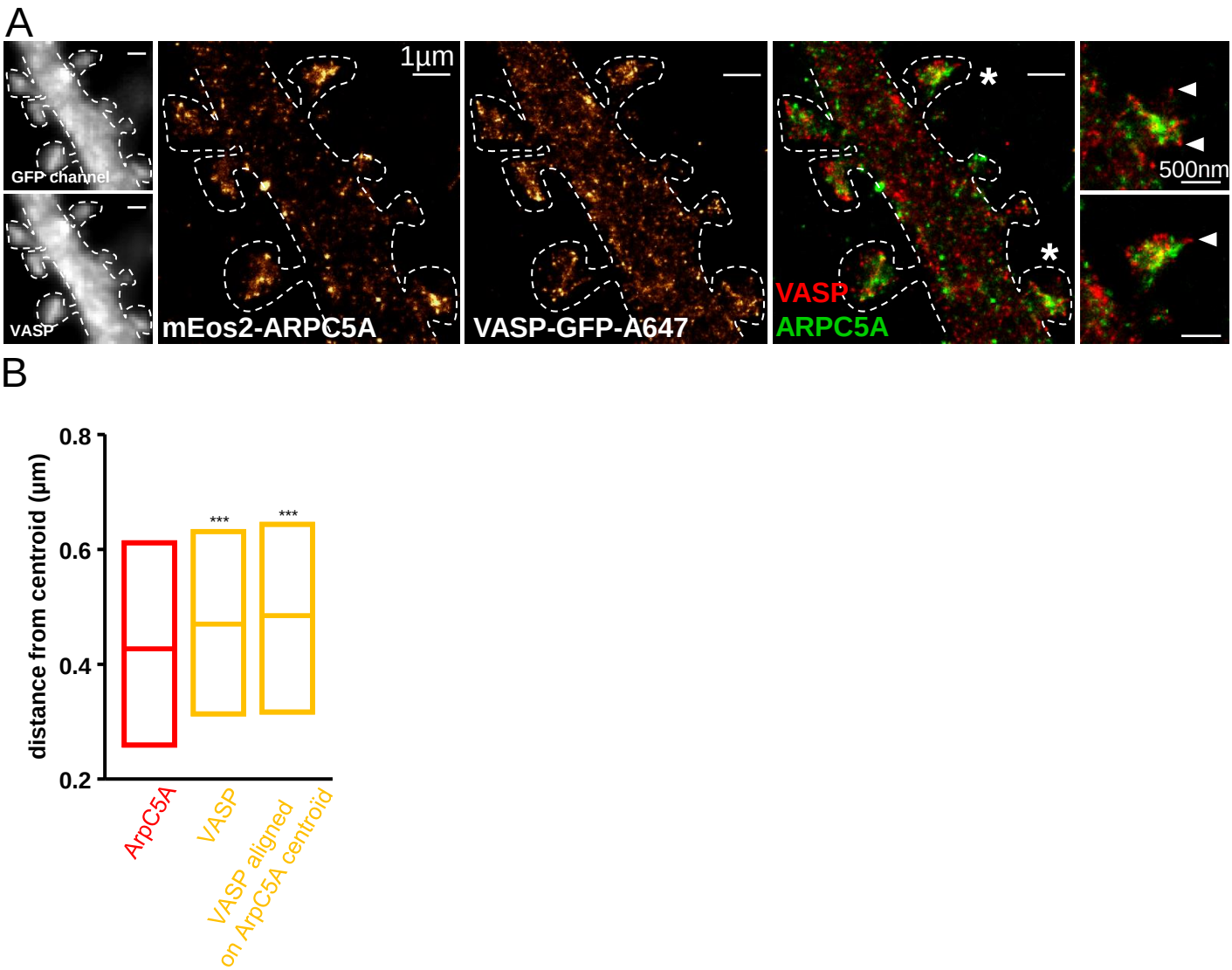
3) It is not clear for me which IRSp53 domains the small circles represent. If they represent IRSp53 membrane binding domains (F-BAR), why are they directed outwards from membrane and not against the membrane?

The small circles represent the SH3 domain of IRSp53 interacting with the proline-rich sequence of WAVE and not the F-Bar domain.



Referee Figure 1: cLTP protocols in dissociated hippocampal neuron.

(A) Fluorescence images of neurons transfected with EGFP or actin-GFP. Two different cLTP stimulation protocols were tested. For the first protocol (middle panels) we applied 200 µM glycine, 5 µM picrotoxin for 3-5 mins in the presence of extracellular Mg^{2+} (Dupuis et al., EMBO J 2014). To induce a stronger activation of NMDA receptors we performed a second cLTP protocol (lower panels) in which we applied 200 µM glycine, 20 µM bicucullin, 3 µM of strychnin for 10 mins in the absence of Mg^{2+} (Fortin et al., J Neurosci 2010). The same neurons were imaged before (left panels) and 30 min after cLTP induction (right panels). For control conditions (upper panels) the neurons were kept in the same imaging solution. Scale bars, 5 µm. **(B)** Box plots displaying the median ± percentile (25-75%) of normalized spine area. Quantifications of spine area before and after cLTP protocols, using EGFP or actin-GFP as a reporter for spine shape, revealed no significant changes. Where indicated, statistical significances were obtained using non-parametric, two-tailed Mann-Whitney rank sum test. **(C)** and **(D)** To test if the second cLTP protocol was nevertheless inducing functional synaptic plasticity, we verified the increased insertion of GluA1 in spines. **(C)** Fluorescence images of neurons transfected with Homer-1C-RFP (upper panels) and immune-stained for endogenous GluA1 (middle panels) 30 mins after cLTP induction (right panels). Merged images (lower panels). Scale bars, 5 µm. **(D)** Box plots displaying the median ± percentile (25-75%) of normalized synaptic GluA1 intensity. Quantifications show an increase in the amount synaptic GluA1 after cLTP induction. Where indicated, statistical significances were obtained using non-parametric, two-tailed Mann-Whitney rank sum test. The resulting *P* values are indicated as follows: ns for $P > 0.05$.



Referee Figure 2: Arp2/3 is not enriched at the tip of VASP dependant membrane protrusions

(A) Dual color super-resolution images using sequential PALM and dSTORM of respectively mEOS2-ArpC5A (left) and GFP-VASP labeled with Alexa647 (VASP-GFP-A647; middle) in a fixed neuron. Merge (right). Scale bars, 1 µm. Left insets: fluorescence image of GFP-VASP and mEos2-ArpC5A (upper panel) and VASP-GFP-A647 (lower panel). Right insets: merge PALM/dSTORM of the spines highlighted by stars in the merge image, scale bars, 500 nm. Arrows indicate VASP accumulations at protrusion tips. Note that mEOS2-ArpC5A do not colocalize with VASP at protrusion tips. **(B)** Box plots displaying the median (notch) ± percentile (25-75%) of distance distribution from the centroid of detections for mEOS2-ArpC5A (red, 5 cells, 26 spines, 6760 detections), VASP-GFP-A647 (yellow, 5 cells, 26 spines, 11374 detections) and VASP-GFP-A647 aligned on mEOS2-ARPC5 centroïd (yellow, 5 cells, 26 spines, 11374 detections). Where indicated, statistical significances were obtained using non-parametric, two-tailed Mann-Whitney rank sum test; the different conditions were compared with the ArpC5 condition. The resulting *P* values are indicated as follows: *** for $P< 0.001$.

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Thank you for the submission of your revised manuscript. Your article has been re-evaluated by the referees and in agreement with them I am pleased to inform you that it has been accepted for publication in the EMBO Journal.

Thank you again for your contribution to The EMBO Journal and congratulations on a successful publication.

